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Evaluation of Factors Related to Growth of Rift Valley Fever Virus in Suspended Cell Cultures

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The effect of several controlled variables on the peak titer and fold increase of Rift Valley fever virus grown in suspension culture on two variants of Earle's L cell, L-DR and L-MA clone 1-1, was studied. No significant amount of cell-associated virus was found at 24 hr, indicating a release of virus soon after its formation. Mild sonic treatment of the virus produced in serum-free medium increased the infective titer about 10×. This difference was not observed with virus produced in medium supplemented with serum. Peak titer was not affected by medium used during the infection period, by multiplicity of inoculum (MOI), or by initial cell concentration within the test range of 10^4 to 2×10^6 cell/ml. Cell strain employed influenced titer, because the L-DR cell did not produce virus efficiently at low MOI and low initial cell concentration. The time of peak titer and fold replication was dependent on MOI and initial cell concentration. Differences in virus propagation in monolayer and suspension systems are discussed.

Rift Valley fever virus (RVFV) is an arthropod-borne virus prevalent in the Kenya area of Africa. It is a disease that infects domestic animals as well as man. Daubney (4) first successfully cultivated RVFV in tissue culture almost 37 years ago, with blood plasma or serum from infected mice as a source of cells. Saddington (14) was the first to propagate RVFV successfully in a chorioallantoic membrane of 9- to 10-day-old chick embryo. The virus was present in large numbers in membranes and in amniotic fluids and livers from the embryos 5 days postinoculation. It was also successfully propagated in the yolk sac and the chorioallantoic membrane by Kaschula (Ph.D. Thesis, Univ. of Pretoria, South Africa, 1953), who obtained the highest titers of the virus from 8-day-old embryonic eggs inoculated in the yolk sac and harvested 48 hr postinoculation.

RVFV has also been grown in several types of tissue cell cultures (5, 10, 15–17, 19). In addition, both the neurotropic and pantropic variants of RVFV have been titrated successfully via plaque formation on rat sarcoma cells (16), Chang's human liver cells (8), and sheep kidney cells (13). Randall et al. (Fed. Proc. **19**: 219, 1960) used monkey kidney cells to produce a vaccine to RVFV.

Orlando et al. (12), of these laboratories, reported that the optimal virus input multiplicity was approximately 2.5. More recently, Johnson et al. (9), using a monolayer tissue culture system of mouse fibroblast-like cells, described the growth of RVFV and reported on optimal virus input multiplicities and medium volume for this system. They also developed and described growth curves for both monolayer and suspension systems.

To date, however, sufficient information has not been available to define the optimal conditions for the propagation of RVFV in a suspension system. In this paper, we describe studies designed to provide sufficient data for the determination of optimal conditions for RVFV in such a system. The major parameters studied were tissue cell lines, concentration, media, and multiplicities of inoculum (MOI). The studies were designed so as to make it possible to determine both the main effects and the interactions among these parameters.

MATERIALS AND METHODS

Viral strain. A small-plaque variant of the pantropic Van Wyk strain of RVFV, isolated by Boyle (2) and grown in cultures of L cells, was used as the primary seed stock. The working virus stock was developed by two passages from the original isolation. The virus suspension contained 10^7 to 10^8 mouse intracerebral lethal doses ($MICLD_{50}$) and was stored in 2-ml amounts at −175 C.

Viral assay procedures. Suspension fluid from virus-infected culture was removed, and 10-fold serial dilutions were made in a mixture of one part medium 199 and two parts Hanks' balanced salt solution (v/v)

supplemented with 10% calf serum. Swiss Webster strain Fort Detrick white mice, weighing 10 to 14 g, were inoculated intracerebrally with 0.03 ml of viral dilution. Eight mice were inoculated per dilution. Deaths were recorded during the next 6 days, eliminating deaths that occurred during the first 24 hr, which were assumed to be traumatic. The probit method (7) of calculating $MICLD_{50}$ values was used.

Disruption of cells by sonic treatment. The Branson Sonifier (model W140C; Branson Instruments, Inc., Melville, N.Y.) was used. Cell suspension (5.0 ml) was placed in a Falcon plastic tube (12 by 75 mm) and cooled in tap water. The sonic oscillator probe was submerged in the cell suspension, and, except as noted in the text, the sonic treatment given was for 20 sec at 20 kc and an input of 10 w. Over 99% of the cells were disrupted by this treatment.

MOI. MOI values were determined by the following formula: volume of viral inoculum to be used = $(A \times B \times C)/D$, where A is the tissue cell count per ml, B is the volume of tissue cells, C is the MOI based on $MICLD_{50}$ desired, and D is the virus titer expressed as $MICLD_{50}$ per ml.

Tissue cell strain. A variant (L-MA) of Earle's L cell was obtained from Donald J. Merchant, University of Michigan, Ann Arbor, Mich. These cells were maintained antibiotic-free, and a selected clonal line designated L-MA Cl 1-1 was established and used as one of the tissue lines in this study. The second variant of Earle's L-cell used was the L-DR line isolated and described by Daniels et al. (3).

Tissue culture procedure. The Merchant spinner flask (Quality Glass Apparatus, Inc., Ann Arbor, Mich.) was used for growth of tissue cells. All cultures were grown in antibiotic-free medium. The growth medium for the L-MA Cl 1-1 cell was medium 199 supplemented with 0.5% peptone (Difco). Medium used to propagate the L-DR tissue cell line was composed of Eagle's minimum essential medium (EMEM) as modified by Daniels et al. (3). Media were supplemented with 10% bovine serum as noted. Viability of cells was determined by the erythrosin B exclusion method, and all cultures used had a viability $\geq 95\%$. Cells were free from contamination by pleuropneumonia-like organisms (PPLO), as determined by repeated failure to detect these organisms on PPLO agar. Bacterial contamination was checked by suitable agar and suspension culture techniques. Throughout these studies, the tissue cells were routinely harvested near the peak of the log phase.

Infection of cultures. Before infection, tissue cells were concentrated by centrifugation (1,085 $\times$ g for 10 min at 4 C) and resuspended to the desired cell concentration in the test medium. Cultures were adjusted to pH 7.4 before infection. The desired MOI of the virus was seeded directly into suspension tissue culture flasks (18) and incubated on the reciprocal shaker (100 three-inch strokes/min) at 37 C for various periods of time, depending on the experimental design. The cultures were assayed for virus production at 0, 24, 48, 72, and 96 hr unless otherwise indicated by the experimental design.

Experimental studies. The work was divided into four areas of investigation. First, two preliminary experiments were conducted to determine the effects of sonic treatment of the culture and of tissue cell concentration on the titer of the virus. These experiments were followed by two others, based on a factorial design, that examined both the main effects of and the interactions among selected variables on infection and virus propagation in a suspension tissue culture system.

Because RVFV tends to clump or aggregate under certain conditions, thus making sonic disruption a necessity for accurate assays, the first preliminary experiment examined the effects of sonic treatment on virus titer. L-MA clone 1-1 tissue cells, grown either in serum-free or serum-supplemented medium, were used. MOI was 0.01, cell concentration was 6×10^5/ml, and the infection period was 24 hr. The sonic input setting was 10 w for 1 min. After this manipulation, virus suspensions were assayed in mice and $MICLD_{50}$ values were determined. The difference between the test and the control (samples without sonic treatment) was used for determining both the effect of clumping on virus titer and the amount or presence of cell-associated virus.

The second preliminary experiment, designed to test the effect of tissue-cell concentration of RVFV infectivity and production, employed L-MA Cl 1-1 tissue cell line propagated in medium 199 plus peptone and supplemented with 10% bovine serum. Tissue cell concentrations of 2×10^5, 10^6, and 2×10^6 were used; the MOI was 0.01. Viral titers were assayed after 0, 24, 48, and 72 hr of incubation. $MICLD_{50}$ values were calculated and incorporated into the analysis of variance to determine the effect of tissue cell concentration on viral infectivity and, possibly, on other interactions that may have occurred. Growth curves for RVFV in different tissue cell concentrations are presented to illustrate different peak titers and when they occur in a suspension culture system.

The first major experiment was designed to measure both the main effects and the interactions that occur in a suspension tissue culture infected with virus. The experimental design was the Latin square; five variations of each of the three following parameters were tested: (i) tissue cell concentration, (ii) tissue cell medium, and (iii) MOI. The experimental Latin square design is illustrated and the variables are defined in Table 1. $MICLD_{50}$ values were calculated after assays at 0, 24, 48, 72, and 96 hr of viral replication or propagation. For optimization of virus titer, an analysis of variance was performed on the peak titers and the initial titers after inoculation. Both main effects and interactions were calculated, and growth curves were plotted illustrating the various phases of viral replication or growth.

The optimal conditions for viral replication or propagation were determined from these results and incorporated into an unreplicated factorial arrangement evaluating tissue cell line, tissue cell medium, tissue cell concentration, and MOI. The variables are defined and the factorial design is presented in Table 2. $MICLD_{50}$ values were determined after 0, 24, 48, and 72 hr to indicate the virus titer. An analysis of variance was performed to determine main effects and interactions. Growth curves were calculated after de-

TABLE 1. *Experimental design for studying the effect of tissue cell concentration, tissue cell medium, and MOI on the growth of RVFV*[a]

MOI	Tissue cell concn per ml				
	2×10^6	2×10^5	10^5	2×10^4	10^4
1.0	M1[b]	M2	M3	M4	M5
0.1	M5	M1	M2	M3	M4
0.01	M3	M4	M5	M1	M2
0.001	M2	M3	M4	M5	M1
0.0001	M4	M5	M1	M2	M3

[a] The experimental design was repeated for each of the two cell lines studied.

[b] M1: modified EMEM supplemented with 10% bovine serum. M2: medium 199 plus peptone supplemented with 10% bovine serum; commercial powder without $NaHCO_3$ (Grand Island Biological Co., Grand Island, N.Y.). M3: Institute of Tissue Culture, powdered medium without $NaHCO_3$ (Grand Island Biological Co., Grand Island, N.Y.). M4: lactalbumin hydrolysate-yeast extract composed of 100 ml of 10× Hanks' balanced salt solution, 1g of dextrose, 5g of lactalbumin hydrolysate, 1g of yeast extract, and 1 g of $NaHCO_3$; volume brought to 100 ml; sterilized by filtration. M5: medium 199 plus peptone, commercial powder without $NaHCO_3$ (Grand Island Biological Co., Grand Island, N.Y.).

termining the main effects and interactions to indicate peak levels of RVFV infectivity or propagation.

RESULTS

Sonic-dispersed particles of RVFV in suspended system. Preliminary experiments utilizing the monolayer system of propagation indicated that RVFV was unstable in a serum-free medium. Therefore, it was necessary to investigate and determine whether this was also true in a suspension system. RVFV was propagated in a suspension system, with the L-MA Cl 1-1 tissue cell line grown in either serum-supplemented or serum-free media inoculated at 0.01 MOI. Four treatments were compared: the tissue cell-virus suspensions both with and without sonic treatment (Table 3, treatments 1 and 2), the centrifuged culture (i.e., devoid of tissue cells) with sonic treatment (Table 3, treatment 3), and the sonic-treated culture centrifuged and again sonic-treated (Table 3, treatment 4). Two important conclusions were drawn: (i) virus aggregation was indicated since virus titers were increased by sonic treatment in serum-free but not in a serum-supplemented medium; and (ii) cell-associated virus was not indicated since the supernatant fluid contained the same titer of RVFV, regardless of other treatments, whether or not the tissue cells were present. Subsequent experimentation showed that neither the time nor the output of oscillator levels was statistically important, and, in effect, verified treatment.

TABLE 2. *Experimental design for studying the effects of tissue cell line, tissue cell medium, tissue cell concentration, and MOI on the growth of RVFV*

Cell concn/ml	MOI	L-DR			L-MA Cl 1-1		
		M2[a]	M3	M4	M2	M3	M4
10^5	0.1	1[b]	2	3	4	5	6
	0.01	7	8	9	10	11	12
2×10^6	0.1	13	14	15	16	17	18
	0.01	19	20	21	22	23	24

[a] Medium formulation as given in Table 1.

[b] Shake-flask code number.

TABLE 3. *Titer of RVFV as affected by sonic treatment*

Treatment no.	Procedure	Log_{10} $MICLD_{50}$ in medium 199 plus peptone				
		Serum-free	10% Bovine serum			Mean
			Replicate 1	Replicate 2	Replicate 3	
1	Tissue cells + virus; not sonic-treated	5.9	5.0	6.0	5.9	5.6
2	Tissue cells + virus; sonic-treated	7.0	5.0	5.8	5.8	5.5
3	Tissue cells + virus; centrifuged, solids removed; supernatant fluid, then sonic-treated[a]	7.0	5.2	5.7	5.7	5.5
4	Tissue cells + virus; sonic-treated, then centrifuged and solids removed; supernatant fluid again sonic-treated[a]	6.8	5.5	6.2	6.0	5.9

[a] Tissue cells were centrifuged at 1,085 × *g* for 10 min.

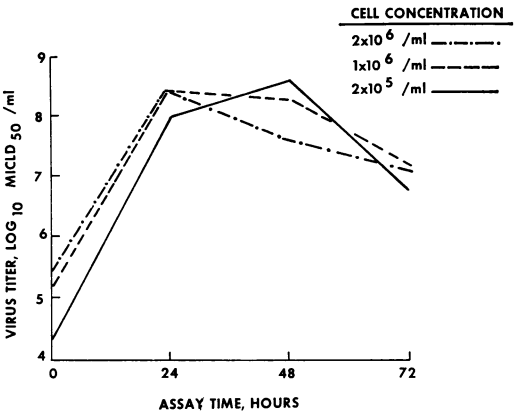

FIG. 1. *RVFV titer through 72 hr for three cell concentrations. MOI, 0.01; tissue cell line, L-MA Cl 1-1; medium 199 plus peptone supplemented with 10% bovine serum.*

TABLE 4. *Analysis of variance for peak titers of RVFV, Latin square design*[a]

Effect	DF	Mean square	F_0	Approximate probability
Cell line (CL)	1	3.645000	2.91	<0.10
Cell concn (CC)	4	9.191875	7.35	<0.01
MOI	4	2.185625	1.75	NS
Media (Med)	4	1.016875	<1	NS
CL × CC	4	0.248125	<1	NS
CL × MOI	4	4.648125	3.71	<0.05
CL × Med	4	1.548125	<1	NS
Error	24	1.251250		
Total	49			

[a]Abbreviations: DF, degrees of freedom; NS, not significant; F_0, mean square of the effect/mean square error.

The knowledge that only a small percentage of virus was cell-associated, along with the effects of sonic treatment in serum-supplemented and serum-free media, provided us with the necessary information for accurate assay of virus infectivity and for further experimental design in optimizing infectivity and growth of RVFV in a suspension culture system. In all later work, sonic treatment for 20 sec at 20 kc and 10 w was used for all virus suspensions.

Effect of tissue cell concentration on infectivity. Different cell concentrations infected with a MOI of 0.01 were assayed for peak titer after 0, 24, 48, and 72 hr postinfection (Fig. 1). Statistical analysis of these data showed that cell concentration had no effect on the titer at 72 hr; however, there was a significant difference between titers at the different assay times ($P < 0.01$). The titers at 24 or 48 hr postinfection were 3 to 4 logs higher than the zero-time titer. Between 48 and 72 hr, the titers declined to approximately the same level; therefore, in a suspension system, cell concentration within the range of 2×10^5 to 2×10^6 does not change the final titer of the culture. It is of interest that a 10,000-fold increase in viral titer was obtained with 2×10^5 cells/ml at 48 hr. Once it was known that final titer did not vary with cell concentration at 72 hr in a suspension system, it appeared desirable to determine the optimal interaction of cell lines, cell concentra-

TABLE 5. *Analysis of variance for fold increases of RVFV, Latin square design*

Effect	DF	Mean square	F_0	Approximate probability
Cell line (CL)	1	10.242340	8.74	<0.01
Cell concn (CC)	4	1.504517	<1	NS
MOI	4	14.135447	12.06	<0.01
Media (Med)	4	1.136287	<1	NS
CL × CC	4	0.517313	<1	NS
CL × MOI	4	3.789493	3.23	<0.05
CL × Med	4	1.465993	<1	NS
Error	24	1.171657		
Total	49			

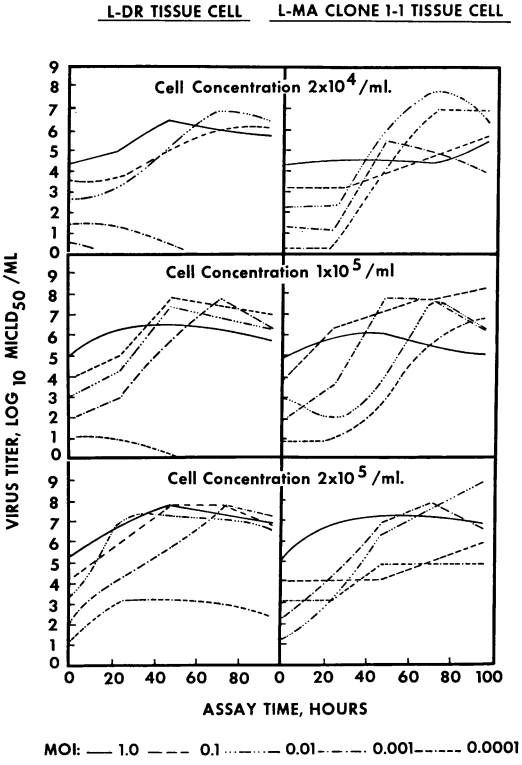

FIG. 2

FIG. 2 and 3. *RVFV curves through 96 hr in two cell lines with different concentrations of cells and MOI; Latin square design.*

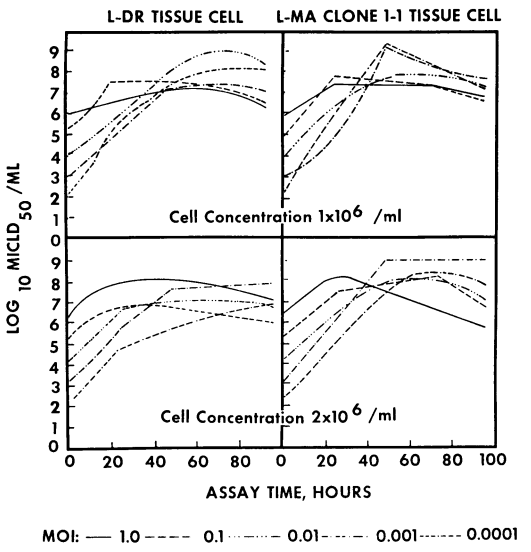

FIG. 3

tions, and MOI in both serum-supplemented and serum-free media.

Latin square design. The Latin square experimental design was used to test a large number of variables that might influence both virus infectivity and replication. The experiment was designed so as to allow the selection of a minimal number of the most promising experimental treatments for subsequent study. Statistical analysis of the data was performed on peak titers obtained at 24, 48, 72, and 96 hr (Table 4). The analysis showed (i) a probable difference between the two tissue cell lines, with L-MA Cl 1-1 having the higher virus yield ($P < 0.10$); and (ii) a significant difference between the tissue cell concentrations ($P < 0.01$). However, there were certain inconsistencies; a cell concentration of 10^5 gave the lowest peak titer, whereas cell concentration of 2×10^5 and 2×10^6 produced the highest peak titers and cell concentrations of 10^5 and 2×10^4 were intermediate. Medium and MOI had no effect on peak titer, and the interaction of tissue cell line with MOI was significant at the 95% level. This difference probably was caused by the low titer for the L-DR tissue cell at MOI values of 0.001 and 0.0001.

Analysis of variance was performed on the increase (log base 2 or fold increase) between initial and peak titer (Table 5), and showed (i) a significant difference among the tissue cell lines tested ($P < 0.01$), with L-MA Cl 1-1 producing the greater fold increase; (ii) a significant difference between the tissue cell concentrations; (iii) a significant difference among MOI values ($P < 0.01$), apparently due to the low virus titer produced by MOI values of 0.001 and 0.0001 in the L-DR line; (iv) no significant difference among media; and (v) a significant interaction between cell lines and MOI at the 95% level. The results of this analysis on fold increase in virus were greatly influenced by the initial virus titer of the various treatment combinations, which ranged from <2.52 to 6.77 for all treatment combinations. Because the type of medium was not significant, values for the media were combined, and the data are presented in Fig. 2 and 3. Virus yield was plotted against assay time at different MOI values, and cell concentrations for the two tissue cell lines were tested. The highest peak titer occurred for both cell lines at a concentration of 10^6 tissue cells per ml. MOI had no effect on the titer reached; however, the time to reach peak titer and the fold replication were dependent on MOI. The only significant interaction was between cell line and MOI for both peak titer and fold replication of the virus ($P < 0.005$). The L-DR tissue cell line produced much lower peak titers at low tissue cell concentrations and low MOI values. Because media had no apparent effect, viral replication apparently proceeded independently, regardless of the type of medium employed, as long as tissue cells were kept viable. These observations were used to determine the variables tested in the factorial experiment.

Factorial design. From results obtained in the preceding experiment, three different media were tested in two tissue cell lines at two different cell concentrations and at two MOI values. This design placed emphasis on optimizing conditions for RVFV replication and growth in the two tissue cell lines. The media for this experiment were selected mainly because they were available in dry form. $MICLD_{50}$ values were determined after 0, 24, 48, and 72 hr and incorporated into the analysis of variance. The results of this analysis (Table 6) showed (i) no significant difference between the two tissue cell lines tested because

TABLE 6. *Analysis of variance for peak titers of RVFV, factorial design*

Effect	DF	Mean square	F_0	Approximate probability
Strain (S)	1	2.9962667	3.65	NS
Media (M)	2	4.5100323	5.50	<0.01
MOI	1	0.6370042	<1	NS
Concn (C)	1	37.0016670	45.11	<0.01
Time (T)	3	52.103220	63.52	<0.01
M × C	2	3.4973136	4.26	<0.05
MOI × T	3	3.5808627	4.36	<0.05
M × MOI × T	6	2.0305427	2.47	<0.05
Pooled 4-way and 5-way interaction[a]	29	0.8202286		
Total	95			

[a] All other interactions were not significant.

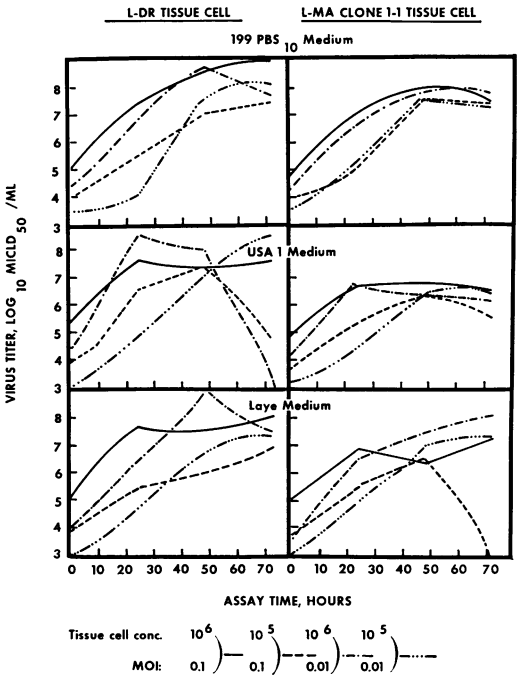

FIG. 4. *RVFV growth curves through 72 hr in two cell lines with different media, cell concentrations, and MOI; factorial design. Media: 199 PBS*$_{10}$*, medium 199 plus peptone supplemented with 10% bovine serum; Grand Island Biological Co., Grand Island, N.Y.; USA1, powdered medium without NaHCO*$_3$ *(Grand Island Biological Co., Grand Island, N.Y.); Laye, lact-albumin hydrolysate-yeast extract.*

higher MOI values were used; (ii) a significant difference between the three media, with medium 199 supplemented with serum producing the highest peak titer ($P < 0.001$); (iii) a significant difference ($P < 0.01$) between tissue cell concentrations, with 10^6 concentration producing the highest titer; (iv) a significant difference among the four assay time periods, with the highest peak titer produced 48 hr postinfection; (v) no significant differences among MOI; and (vi) significant interactions between medium and cell concentration ($P < 0.05$), MOI and time ($P < 0.05$), medium, MOI, and time ($P < 0.05$), and MOI, cell concentration, and time ($P < 0.01$). From this analysis of variance, growth curves were plotted for each medium, combining tissue cell concentration and MOI (Fig. 4). In this factorial experiment, in which significant variables had been selected and their critical level evaluated from the Latin square experiment, smaller differences were detectable. Medium containing serum was significantly better than medium without serum. Perhaps the serum affected virus replication or stability on release from the cell. Again, MOI was not statistically significant, with the higher MOI values producing peak titers at earlier time periods than the low MOI values. Time of peak titer was also affected by cell concentration. Tissue cell concentration again was highly significant. Both tissue cell concentration and MOI affected the time of peak titer, and the concentration of both cells and MOI may be adjusted to effect the desired time of peak production.

DISCUSSION

Generally, the growth curves of RVFV in a suspension system are not unlike the general growth curves developed by Johnson and Orlando (9) for a monolayer system: lag or latent periods, log or exponential growth phases, and stationary phases preceding death phases were observed. The time of initiation and the length of each phase were dependent on MOI and cell concentration. The peak titer attained was not affected by any of the variables studied, indicating that inhibition or possibly inactivation of the virus takes place at approximately the same peak level of growth. The fact that the mean fold replication of virus varied by 2.8 logs but still reached the same peak titer supports the probability that viral replication stops because of inhibitory interaction between virus and tissue cells.

Our experiments indicate that the significant differences between growth of RVFV in suspension and growth in monolayer cultures as described by Johnson and Orlando are (i) the lack of cell-associated virus in suspension culture and (ii) the initiation of infection and growth to the same peak titer at very low MOI values. We suggest that the ability of low MOI values to reach the same peak titers may be attributed to the fact that cells and virus in suspensions are constantly in motion and, consequently, have multiple contacts and possibilities of cell attachment, but movement of virus in a monolayer system is essentially Brownian motion (1).

The lack of cell-associated virus in suspension culture indicates that the virus is released to the medium soon after its formation within the tissue cell. This observation contrasts with the findings of Johnson and Orlando (9), but is supported by unpublished work of L. E. Schneider, of our laboratories, who was unable to demonstrate virus aggregates within the cell by use of fluorescent ribonucleic acid (RNA)-specific staining techniques (*personal communication*).

If the virus is released into the culture medium soon after its formation in the tissue cell, the effect of the culture medium and environment on virus stability or inactivation becomes very important. Our results show that serum increases stability of the virus for a long period of time after its release.

Increases in peak virus titer as high as 10-fold

were attributable to sonic treatment of virus produced in serum-free medium, a difference which was not observed when RVFV was propagated in a serum-supplemented medium. Since inactivation titer resulting from sonic treatment is probably attributable to a change in viral aggregate or molecular properties.

The L-DR cell did not produce virus when MOI values of 0.0001 were used with cell concentrations of 2×10^4, 10^5, and 2×10^5 or when an MOI of 0.001 was used with a cell concentration of 2×10^4, whereas the L-MA Cl 1-1 line produced virus to the same titer as higher MOI values or cell concentrations in all these cases. The L-DR cell was grown in serum-supplemented medium, whereas the L-MA Cl 1-1 line was not. This difference in cell lines could be genetic; however, we believe it most probably is a physiological or morphological change attributable to serum, which affects either viral attachment sites or possibly both attachment sites and replication.

From the growth curves established and the great number of variables tested, it appears that optimal growth of RVFV in the suspension system is dependent on the tissue cell line only, irrespective of MOI, tissue cell concentration, and tissue cell medium employed. Moreover, for optimal yields within a tissue cell line, the only limiting factor is the incubation time, which is entirely dependent on the MOI and cell concentration used in a suspension tissue culture system. Melnick (11), working with poliomyelitis virus, also noted that peak titer was not affected by inoculum size; however, the time at which the peak titer developed was dependent on the size of inoculum. It is also apparent from our observations that serum stabilizes the infectious virus particle over prolonged periods of incubation. Therefore, if a serum-free medium is used during infection, then a high MOI and a high cell concentration should be used to shorten the incubation time. It is also apparent that RVFV can be grown efficiently in a suspension system, producing high yields of infectious RVFV particles. Such a system offers potential for other arboviruses in the stimulation of high virus yields for vaccine production and in the concentration of infectious RNA for chemical and genetic evaluation.

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